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Nativelike Enzyme Properties Are Important for Optimum Activity in Neat Organic Solvents

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It has been suggested that evolutionary adaptation has adjusted enzyme conformational mobility as a key parameter allowing optimum catalysis at a given temperature.¹ For example, the concept of “corresponding states”^{1c} for homologous enzymes from mesophilic and thermophilic organisms states that enzyme topology, flexibility, and mechanism are comparable at the temperature where the enzymes display optimum activity.^{1b} We investigated whether this concept would hold true for enzymatic catalysis in organic solvents. We demonstrate that the highest enzyme activity was displayed when the structure and conformational mobility was most similar to that of the enzyme in aqueous buffer. This suggests that the evolutionary adaptation concept¹ may indeed be applicable to enzyme activity and activation in neat organic solvents.

For dehydrated enzyme powders suspended in organic solvents molecular lubricants² are critical for achieving an optimum enzyme activity.³ For example, adjustment of the level of residual enzyme bound water is important for attaining optimum enzyme activity in organic solvents,⁴ which strongly suggests a relationship between enzyme activity and conformational mobility in organic solvents.^{3,4e} This is also supported by enzyme activation with denaturing cosolvents, such as DMSO and dimethyl formamide,⁵ and crown ethers⁶ and cyclodextrins.⁷ We have demonstrated that the latter macrocyclic compounds increase the conformational mobility of the suspended enzyme.^{6e,7a}

Identification of factors contributing to optimum enzyme activity in organic solvents is notoriously difficult.^{3,6e,7a,b} This is due to the fact that the drop in activity when compared to the aqueous environment may stem from changes in protein structure and dynamics, energetics of substrate desolvation and transition state stabilization, among other.³ We pursued the strategy to vary the conformational mobility and structure of subtilisin Carlsberg in 1,4-dioxane by (a) co-drying with various concentrations of the crown ether 18-crown-6 and (b) addition of DMSO. Thus, we only changed one experimental parameter in a set of

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Supporting Information Available: (1) Calculation of the spectral correlation coefficient, second derivative spectra of subtilisin films (2) with 18-crown-6 and (3) in 1,4-dioxane/DMSO at various temperatures, (4) plots of the spectral correlation coefficient vs temperature used to determine the T_d values, (5) tables with the correlation coefficients for the various subtilisin films prior to and after solvent exposure at various temperatures (PDF). This material is available free of charge via the Internet at <http://pubs.acs.org>.

experiments. Monitoring of subtilisin secondary structure and measurement of the thermal denaturation temperature (T_d) by Fourier transform infrared (FTIR) spectroscopy⁸ allowed for pinpointing the effects of these critical properties on enzyme activity.

The formation of the *S* product ester^{9,10} in the reaction of *sec*-phenethyl alcohol with vinyl butyrate^{6e,7a,b} was studied for subtilisin co-dried with increasing concentrations of 18-crown-6. After initial enzyme activation (Figure 1A) with an optimum activity at the 0.7 mass ratio (crown ether-to-subtilisin), the activity dropped. Such curves have been reported before,^{6d} but the reason for the activation/inactivation pattern is still largely speculative. To relate the observed activity to subtilisin structure and conformational mobility, FTIR spectroscopic studies were conducted. Subtilisin secondary structure was markedly influenced by the concentration of the crown ether (Figure 1B) and was most nativelike at crown ether-to-subtilisin mass ratios of 0.7:1 and 1:1. At lower ratios the structure was more similar to that found for subtilisin dried without the crown ether and exposed to 1,4-dioxane, at higher ratios some solvent-induced denaturation occurred as evidenced by the intensity increase of IR bands typically associated with the formation of intermolecular β -sheets.¹¹ Thermal denaturation of subtilisin in the crown ether containing films^{6e} lead to the formation of intermolecular β -sheets in all cases studied. The denaturation temperature (T_d) was determined from plots of the spectral correlation coefficient¹² versus the temperature.^{6e,7a} The T_d -values (Figure 1A) decreased with increasing crown ether concentration, symptomatic of a less rigid protein structure.^{3b,13} These results suggest that the optimum enzyme activity displayed by the film obtained at the 0.7:1 mass ratio corresponds to an overall enzyme conformational mobility characterized by a T_d of ca. 68 °C.¹⁴ This value is close to that found in aqueous solution of 73 °C.^{8a} If the initial concept were to hold true,¹ one would expect that the highest enzyme activity should be found for the film with the 0.5 mass ratio (T_d 70 °C). However, the structure of the enzyme catalyst cannot be neglected in this context,^{3,7a} and subtilisin secondary structure was more nativelike for the sample obtained with the 0.7 mass ratio (Figure 1B). Thus, the sample that had both (similar secondary structure and similar rigidity properties as subtilisin in water) had the highest activity.

To test the generality of our findings, a second set of experiments was conducted using a different system. We examined the activation of subtilisin in 1,4-dioxane at increasing concentrations of the denaturing solvent DMSO.⁵ The kinetic data obtained (Figure 2A) show an optimum subtilisin activity at 5–10% of DMSO in 1,4-dioxane (vol/vol) followed by a substantial drop in activity at higher DMSO concentrations. However, the enzyme activation was substantially less pronounced than with 18-crown-6. Thermal denaturation resulted in the unfolding and formation of aggregation β -sheets. With the exception for the data point obtained at 5% DMSO with a T_d of 84 °C, the T_d values were 61–68 °C for the other concentrations and thus the enzyme less rigid than in the aqueous environment (Figure 2B). The secondary structure of subtilisin dried as films and exposed to 1,4-dioxane did change significantly with increasing DMSO concentrations. Subtilisin was most nativelike in films exposed to low concentrations of DMSO in 1,4-dioxane (5% and 10%, respectively). Subtilisin structure was more nativelike in these samples than when exposed to 1,4-dioxane alone. Clearly, the additive DMSO allowed for refolding of dehydrated

subtilisin supporting the notion of a kinetically less constraint protein structure.¹⁵ This case represents yet another example that neat organic solvents do not necessarily cause protein unfolding, but quite the opposite, protein refolding.¹⁶ At higher DMSO concentrations (in particular, at 30% DMSO), subtilisin structure was increasingly perturbed.

The data demonstrate again that optimum activity coincided with subtilisin structure and rigidity most similar to those of subtilisin in water. The highest activity was observed for the samples in 1,4-dioxane between 5 and 10% of DMSO. However, there must also be other factors responsible for subtilisin activation in 1,4-dioxane because of the substantial differences in activation achieved with 18-crown-6 and DMSO. Even though enzyme structure and rigidity were comparable for some data points close to the maximum activity values in both experiments, enzyme activation by 18-crown-6 was substantially higher. A likely explanation for this is the reported molecular imprinting effect exerted by 18-crown-6 substantially contributing to enzyme activation.^{6d,e}

In summary, while our results do not exclude other factors from contributing to the activity and activation of enzymes in organic solvents,^{3b,6e} we demonstrate for the first time that the concept of corresponding states¹ can indeed be extended to enzymes in neat organic solvents.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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8. FTIR experiments (including the thermal denaturation measurements) were conducted and analyzed as described in detail by us.^{6e,7a,b} In short, subtilisin was dried as thin film on CaF₂ FTIR windows and exposed to 1,4-dioxane. FTIR spectra were measured at various temperatures. Overall structural perturbations were quantified by calculating the spectral correlation coefficient between the second derivative amide I spectra of the sample at ambient temperature vs elevated temperature. From plots of r vs temperature, the denaturation temperature T_d was obtained from the minimum of the first derivative of a sigmoid function fitted to the data.
9. Subtilisin films were prepared, kinetic measurements performed and analyzed by chiral GC as described by us in detail.^{6e}
10. Subtilisin enantioselectivity remained constant independent of the crown ether concentration with values for E^{6e} between 17 and 21.
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14. For enzymes in organic solvents H/D exchange data are not revealing in this context because they are influenced by protein structural and dynamic properties. In our case H/D exchange data would vary for most samples because of structural perturbations (see Figures 1B, 2B).
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Figure 1.

(A) Initial rate of the formation of S-product ester in the reaction of *sec*-phenethyl alcohol with vinyl butyrate in 1,4-dioxane (mg in unit for V_S refers to pure enzyme), (B) T_d determined by FTIR spectroscopy (◇), and similarity of subtilisin structure at 45 °C with that of native subtilisin (○) for subtilisin co-dried with 18-crown-6 in 1,4-dioxane. The horizontal dotted line in (B) represents the T_d for subtilisin in aqueous buffer, the vertical one the mass-ratio with optimum activity.

Figure 2.

(A) Initial rate of the formation of S-product ester in the reaction of *sec*-phenethyl alcohol with vinyl butyrate (mg in unit for V_S refers to pure enzyme), (B) T_d determined by FTIR spectroscopy ($\diamond$), and degree of similarity of subtilisin structure at 45 °C with that of native subtilisin ($\circ$) for subtilisin films exposed to 1,4-dioxane/DMSO mixtures.